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Processing of unstable bacteriophage T4 gene 32 mRNAs into a stable species requires *Escherichia coli* ribonuclease E

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Gene 32 from bacteriophage T4 is transcribed as precursor transcripts which are processed to a stable product. This processing of the gene 32 mRNA was observed in RNase III or P-deficient strains of *Escherichia coli*. However, after infection of an RNase E-deficient strain, the amount of processed transcript was significantly reduced while the levels of the precursor transcripts remained high. RNase E therefore appears to have an essential role in the processing of the gene 32 mRNA. We have mapped the exact 5′ end of the processed transcript by primer extension. The cleavage occurs near a stem–loop structure at a site which shows some similarity to other known RNase E cleavage sites. The effects of the processing on the differential stability of the upstream and downstream sequences, and on gene expression, are discussed.

Key words: mRNA processing/promoters/ribonuclease E/T4 gene 32/transcription starts

Introduction

Bacteriophage T4 gene 32 encodes the single-stranded DNA-binding protein which is essential for replication, recombination and repair of T4 DNA (Alberts and Frey, 1970). A remarkably complex control mechanism exists to ensure an adequate supply of this protein in response to the infected cell's requirements. One unusual feature of this control is that the expression of the gene is self-regulated at the level of translation (Lemaire *et al.*, 1978; Krisch and Allet, 1982). In addition, its transcription is temporally regulated by successive phage-controlled modifications of the host RNA polymerase. Prior to the onset of DNA replication, it is transcribed from promoters for polycistronic and monocistronic messages whose recognition is facilitated by the phage-encoded product of the *mot* gene (Mattson *et al.*, 1978; Uzan *et al.*, 1985; Belin *et al.*, 1987). The polycistronic message encodes several upstream genes (see Gorski *et al.*, 1985). Once DNA replication commences, the *mot*-activated transcription is turned off and a monocistronic transcript is initiated at a phage late promoter. The 5′ ends of the primary monocistronic transcripts are located approximately at positions −200 (pre-replicative transcript) and −150 (late transcript) relative to the gene 32 translation initiation codon. Both of these ends were substrates for the vaccinia capping enzyme, confirming that they represent the starts of primary transcripts (Belin *et al.*, 1987).

Gene 32 monocistronic mRNAs can be very stable (Gorski *et al.*, 1985). Interestingly, it was found that the larger, less stable gene 32 transcripts were processed to a stable product whose 5′ end was located approximately at position −75. Since the 5′ end of the processed species was detected in uninfected cells carrying hybrid plasmids of the gene 32 leader sequence, it was suggested that a host ribonuclease was responsible for the processing reaction (Belin *et al.*, 1987).

Unlike eukaryotes, where mRNA processing as a means of post-transcriptional gene regulation is common and well documented, only a few examples of mRNA processing in prokaryotes have been found. The roles of *Escherichia coli* endo-ribonucleases III and P in the maturation of ribosomal and transfer RNAs respectively have been characterized, but only RNase III has been shown to have a role in mRNA processing (for reviews, see Gegenheimer and Apirion, 1981; King and Schlessinger, 1987). The involvement of *E.coli* RNase E in the processing of 9S RNA to a precursor of 5S rRNA has been established (Ghora and Apirion, 1978; Misra and Apirion, 1979; Roy *et al.*, 1983). This enzyme also processes RNA1, the inhibitor of ColE1 plasmid replication (Tomcsányi and Apirion, 1985). The enzyme has been partially purified and some of its characteristics have been determined (Misra and Apirion, 1979; Roy *et al.*, 1983). From a comparison of the cleavage sites for the 9S RNA and RNA1 substrates, a potential RNase E recognition sequence was proposed (Tomcsányi and Apirion, 1985).

In this communication we present evidence that host-encoded RNase E is essential for the processing of the gene 32 mRNAs. We have also identified the precise 5′ ends of the gene 32 primary monocistronic transcripts and of the processed transcript. This is the first report of RNase E being involved in the processing of a messenger RNA.

Results

Determination of the precise 5′ ends of the primary and processed gene 32 transcripts

We have shown previously that, in T4-infected cells, the 5′ ends of the gene 32 monocistronic mRNAs are at ~200, 150 or 75 nt from the gene 32 translation initiation codon (Belin *et al.*, 1987). These 5′ ends had been mapped by complementary RNA (cRNA) protection experiments. The −75 transcript represented a processed product of the larger transcripts. Since mRNA processing appeared to be an important feature of gene 32 expression, we were interested in determining the precise site of cleavage in the primary transcripts. Furthermore, a knowledge of the exact primary structure of the processed transcript could be important for understanding why this RNA is stable. The 5′ ends of the gene 32 mRNAs were therefore mapped to the nucleotide by primer extension (Figure 1). Total RNA from phage-infected cells was isolated, annealed to a gene 32-specific

oligodeoxynucleotide primer (see Materials and methods), copied by reverse transcriptase and electrophoresed alongside dideoxy sequencing reactions.

The 5′ end of the processed transcript is actually 71 nt upstream of the gene 32 initiation codon (Figure 1, panel A, lane 2). The sequence shown in Figure 1 is the complement of the mRNA, so the first nucleotide of the processed transcript is an A. Panel B of Figure 1 shows the 5′ ends which are further upstream. The 5′ end of the late transcript (lane 1) is the complement of the C at position −145, while that of the prereplicative early transcript is complementary to the A at position −205 (lane 2). Figure 2 shows the locations of the 5′ ends of the gene 32 transcripts in the nucleotide sequence of the gene 32 leader region.

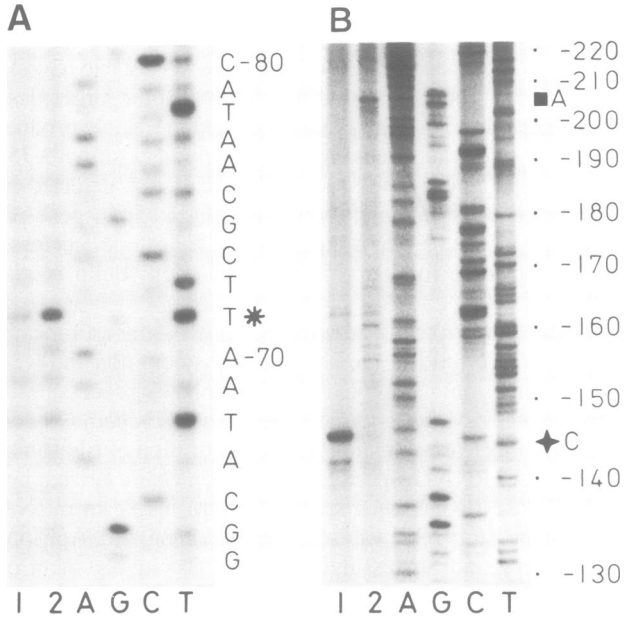

Fig. 1. The precise 5′ ends of the gene 32 transcripts. RNA was extracted from cells infected at 37°C, hybridized to the primer, extended with reverse transcriptase and electrophoresed on a sequencing gel alongside the gene 32 leader sequencing ladder. **Lane 1**, 15 min after infection with a wild-type phage; **lane 2**, 10 min after infection with a $32^{-}44^{-}$ mutant phage. This mutant phage is blocked for late gene expression and derepressed for gene 32 protein synthesis. Lanes marked A, G, C and T represent the dideoxy sequencing reactions. **Panel A** shows the sequences around the −71 processing site, while **panel B** encompasses the 5′ ends of the larger −205 and −145 transcripts from the upper portion of the same gel. The nucleotide sequence given is that of the gene 32 coding strand. The coordinates refer to the positions of the upstream sequences relative to the A of the gene 32 initiation codon at +1. *, ✦ and ■ represent the complements of the first nucleotides of the −71, −145 and −205 transcripts respectively. Guild *et al.* (1988) have localized the 5′ end of the −205 transcript to this position, or the C at −204.

The levels of each of these transcripts in a wild-type and a mutant phage infection were examined to ensure that their 5′ ends represent the same ends which had been mapped previously, but less precisely, by the cRNA protection experiments (Belin *et al.*, 1987) rather than being artefacts caused by *in vitro* stops of the reverse transcriptase. The level of the −71 transcript at late times after infection is much higher in the mutant phage infection (for details of this mutant phage see legend to Figure 1) than in the wild-type infection (cf. lanes 1 and 2, panel A). This is the expected result for the processed transcript based on the previous observations. Likewise, the transcript with the 5′ end at position −145 must be the late transcript since it is barely detectable in the mutant phage infection but is abundant late in a wild-type infection (cf. lanes 1 and 2, panel B). Finally, as expected, the early −205 transcript is detected in the RNA from the mutant phage infection (panel B, lane 2) but not in the RNA from a late time point in a wild-type infection (panel B, lane 1). The use of these two different infections in the primer extension experiments therefore confirm that the 5′ ends of each of the transcripts are equivalent to those mapped previously by cRNA protection.

The effect of host ribonuclease mutations on the processing of the gene 32 mRNAs

We had previously suggested that a host-encoded ribonuclease was responsible for the processing of the gene 32 mRNA (Belin *et al.*, 1987). Since RNases P, III and E are known to be involved in RNA processing in *E.coli*, we examined the effects of temperature-sensitive mutations in the genes encoding these enzymes on the cleavage of the gene 32 transcripts. The phage used to infect these RNase-deficient strains was defective for a late gene transcription factor (the gene 55 product) (Bolle *et al.*, 1968), since the amount of the processed species was much higher in this

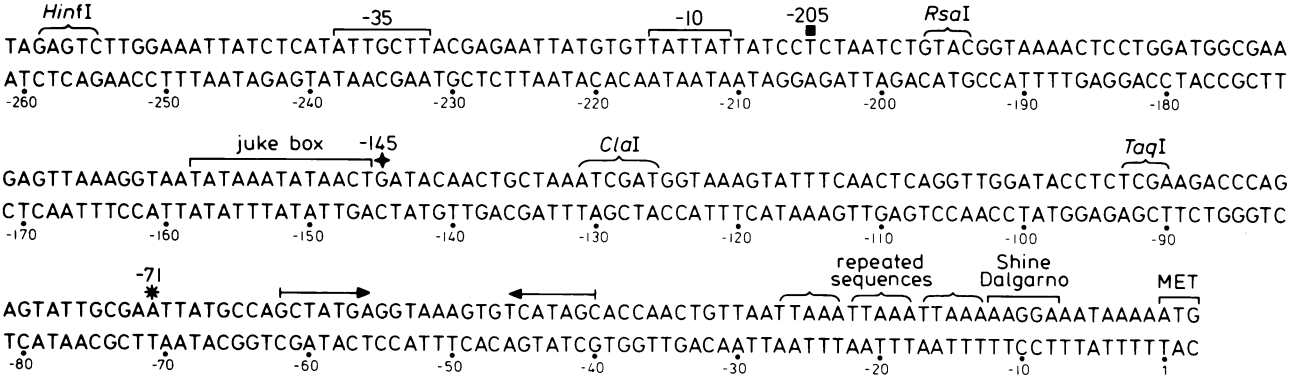

Fig. 2. Nucleotide sequence of the gene 32 leader region showing the locations of the 5′ ends of the primary and processed monocistronic phage transcripts. The nucleotide sequence data are from Krisch and Allet (1982). The first nucleotides of the −205, −145 and −71 transcripts are indicated as ■, ✦ and * respectively. The positions of the −35 and −10 sequences of the *mot*-activated promoter for the −205 transcript are shown. The −35 sequence (ATTGCTT) is very similar to the consensus −35 sequence for *mot*-activated promoters (Brody *et al.*, 1983). The consensus sequence for the late promoter (−145) (Belin *et al.*, 1987), also known as the 'juke box' sequence (Christensen and Young, 1983), is indicated. The repeated sequences immediately upstream of the Shine−Dalgarno sequence have been implicated in the translational self-regulation of gene 32 (Krisch and Allet, 1982). The inverted repeat sequence of the stem−loop structure (see Figure 6) is also indicated.

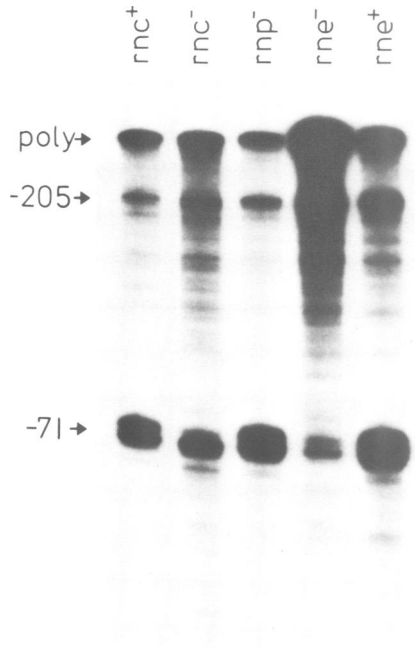

Fig. 3. The 5′ ends of the gene 32 transcripts in *rnc*$^+$ and *rnc*$^-$, *rnp*$^-$, *rne*$^-$ and *rne*$^+$ strains of *E.coli* infected with a 55$^-$ mutant phage. RNA isolated at 20 min after infection with a 55$^-$ mutant phage at 43°C was analysed by cRNA protection (Materials and methods). The samples were electrophoresed on a 9% polyacrylamide−7 M urea gel. The locations of the polycistronic ('poly', representing full-length protection of the cRNA) transcript, and the 5′ ends of the −205 and −71 transcripts are shown. Twice as much total RNA was used for the *rnc*$^-$ strain due to the reduced ability of T4 to infect it (see Materials and methods). The apparent splitting of the −71 transcript into two bands of similar sizes observed here is dependent upon the concentration of pancreatic RNase, and is due to incomplete digestion.

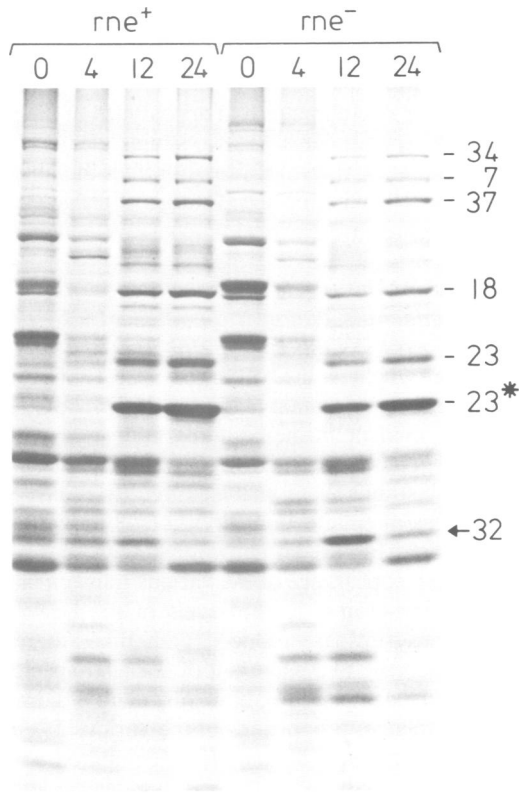

Fig. 4. Protein synthesis during the course of a wild-type phage infection of *rne*$^+$ and *rne*$^-$ strains of *E.coli*. The ^{14}C-pulse-labelled proteins synthesized at 43°C during a wild-type phage infection of *rne*$^-$ and *rne*$^+$ strains, at the times indicated, were analysed by SDS−PAGE using a 10% polyacrylamide gel. Equal volumes of cell lysates, which contained equivalent amounts of total proteins as shown by Coomassie blue staining of the gel, were loaded into each track. The gene 32 polypeptide is indicated by an arrow. Some T4 late gene products (Vanderslice and Yegian, 1974) are also indicated.

mutant phage infection than in a wild-type infection (Belin *et al.*, 1987). Following infection of the *E.coli* RNase mutant strains with the 55$^-$ mutant phage, RNA was extracted from the cells and hybridized with *in vitro* synthesized ^{32}P-labelled cRNA to the gene 32 leader region (from positions −343 to +3). After digestion of the non-hybridized RNA with RNase, RNA duplexes were denatured and the protected ^{32}P-labelled strands were analysed by gel electrophoresis and autoradiography.

The pattern of the 5′ ends in the RNase E-deficient (*rne*$^-$) strain is strikingly different from that in the isogenic wild-type (*rne*$^+$) strain and from the other mutant and wild-type strains (Figure 3). The level of the processed −71 transcript is significantly reduced, while the levels of the precursor transcripts are much higher. This is in contrast to the situation found in the RNase P-deficient (*rnp*$^-$) or the wild-type strains (*rnc*$^+$ and *rne*$^+$) where the levels of the −71 transcripts are higher than the levels of the precursor polycistronic (poly) and −205 transcripts. The RNase III-deficient strain (*rnc*$^-$) shows a slightly reduced level of the −71 species and slightly higher levels of the precursor transcripts at this time point compared with the *rnc*$^+$, *rnp*$^-$ and *rne*$^+$ strains. However, in comparison to the other strains used in Figure 3, the appearance of the 5′ ends of the gene 32 transcripts is delayed in this strain (data not shown). The results of the experiments with the three RNase-deficient strains therefore suggest that RNase E, but not III or P, is essential for the processing of the gene 32 mRNA.

Since it was possible that the effect of the *rne* mutation on the gene 32 mRNA processing was due, for example, to a major alteration of phage development, the pattern of protein synthesis in this host following infection with a wild-type phage was examined. During a normal wild-type phage infection, the synthesis of host proteins is turned off and the phage directs the synthesis first of 'early' proteins such as those required for phage DNA replication, and subsequently of 'late' proteins which are mainly concerned with phage assembly. Figure 4 shows the proteins synthesized before and after a wild-type phage infection of the temperature-sensitive *rne*$^-$ mutant and isogenic *rne*$^+$ strains at the nonpermissive temperature. Gitelman and Apirion (1980) had previously shown that a mutation in the gene for RNase E affects the synthesis of some cellular proteins. This difference can also be seen in the '0' lanes of Figure 4, but it is not seen at the permissive temperature (data not shown). At 12 min after infection it is evident that the level of synthesis of the gene 32 protein is somewhat higher in the *rne*$^-$ mutant than in the wild-type strain. This effect may be due to the increased levels of the poly and −205 transcripts in the mutant strain at this time (data not shown). Alternatively, this may reflect slight differences in the kinetics of phage development. The synthesis of the phage late proteins is delayed to a small extent in the *rne*$^-$ mutant

strain, and this could in turn delay the reduction in the synthesis of the gene 32 protein normally observed at late times of infection and evident in the *rne*$^+$ infection. Nevertheless, the overall pattern of appearance of the phage late proteins is very similar in the two strains, demonstrating that the *rne* mutation does not grossly affect the ability of T4 to infect the cell and direct the synthesis of phage-encoded proteins.

A comparison of the levels of the precursor and processed transcripts during the course of infections of RNase E$^+$ and RNase E$^-$ strains

To determine whether the observed effect of the *rne* temperature-sensitive mutation on the level of processed gene 32 mRNA is evident throughout the course of a phage infection, the 5′ ends of the gene 32 transcripts in the isogenic *rne*$^-$ and *rne*$^+$ strains were analysed by cRNA protection at various times after infection (at 30 or 43°C) with gene 55 mutant phage. Figure 5A shows that at the permissive temperature, the appearance of the various transcripts is the same in the two strains: as the levels of the larger poly and −205 transcripts are reduced with time, there is a concomitant increase in the amount of the −71 species. At the nonpermissive temperature, however, there is a dramatic change in the relative proportions of the various 5′ ends in the RNA from the *rne*$^-$ mutant strain compared with the RNA from the *rne*$^+$ strain. In the *rne*$^-$ strain, the levels of the larger transcripts are not reduced with time, and there is a significant decrease in the level of accumulation of the −71 species.

The extent of the difference in levels of the −71 species 20 min after infection of the two strains was estimated by dilution (Figure 5B). The level in the mutant is between 10 and 20% of that in the wild-type strain, and is detected presumably because the host mutation is leaky. Indeed, some 5S rRNA is produced in *rne*$^-$ strains at the nonpermissive temperature (Apirion and Lassar, 1978; Ghora and Apirion, 1979), which also suggests that this mutation may be leaky. A similar comparison of the levels of the polycistronic and −205 transcripts at this same time point (Figure 5B) shows that the amount of polycistronic transcript is approximately five times higher in the mutant strain while the increase in the amount of −205 is slightly less than this. These results demonstrate that in the RNase E-deficient strain there is a significant reduction in the extent of the processing of the larger transcripts to the −71 species.

It should be noted that in these cRNA protection experiments, only sequences downstream from the processing site were detected. Processing of the polycistronic and −205 transcripts should also yield upstream RNA that would give rise to protected species of 272 and 134 nt respectively. The failure to detect such upstream products (Figures 3 and 5) indicates that, following processing, the sequences upstream of the cleavage site are much less stable than those downstream.

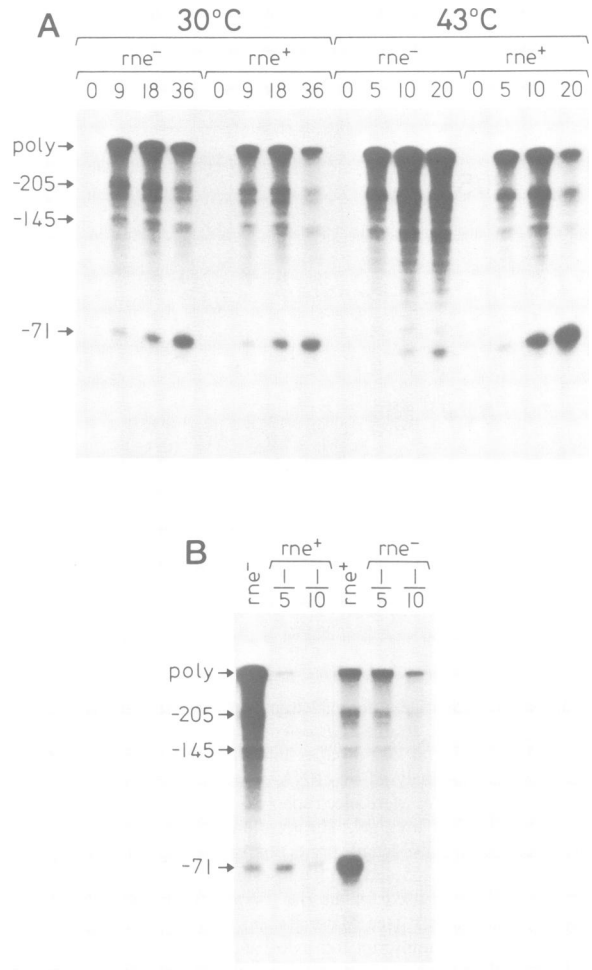

Fig. 5. The 5′ ends of the gene 32 transcripts in *rne*$^-$ and *rne*$^+$ strains infected with a 55$^-$ phage. Analysis of the 5′ ends of the gene 32 transcripts was as described in Figure 3. (**A**) RNA was isolated at the indicated times afer infection of *rne*$^-$ and *rne*$^+$ strains at 30 or 43°C. (The time points used for these two temperatures are approximately equivalent with respect to kinetics of phage development.) The 5′ ends are indicated as described in Figure 3. Note that the −145 late transcript is present at a low level in the RNA from this phage infection in which late transcription is reduced. (**B**) The 20-min time point for the *rne*$^+$ strain at 43°C was diluted as indicated and is shown adjacent to the same time point for the undiluted *rne*$^-$ strain. Similarly, dilutions of the 20-min sample for the *rne*$^-$ strain are shown next to the undiluted sample from the *rne*$^+$ strain.

Discussion

In this communication we have presented evidence that the processing of the gene 32 messenger RNA is dependent upon the host-encoded RNase E. Previous work has implicated RNase E in the processing of the non-coding stable RNAs, 9S RNA and RNA1, but not of mRNA. The products of the processing of these substrates have monophosphate groups at their 5′ ends (Roy *et al.*, 1983; Tomcsányi and Apirion, 1985). The 5′ end of the gene 32 processed transcript can be kinased only after phosphatase treatment (data not shown), implying that it also has a 5′ phosphate group(s). Since this transcript was not a substrate for the vaccinia capping enzyme (Belin *et al.*, 1987), an enzyme which only caps transcripts with 5′ tri- or diphosphate ends, it is very likely that this transcript has a 5′ monophosphate group. This result is consistent with the −71 transcript being a product of RNase E processing.

A comparison of the sequences flanking the cleavage site of the processed gene 32 message to the proposed RNase E 'recognition sequence' (Tomcsányi and Apirion, 1985) (Figure 6) shows that there is only a 5 out of 10 match. However, all the conserved bases (GA/AUU) are clustered

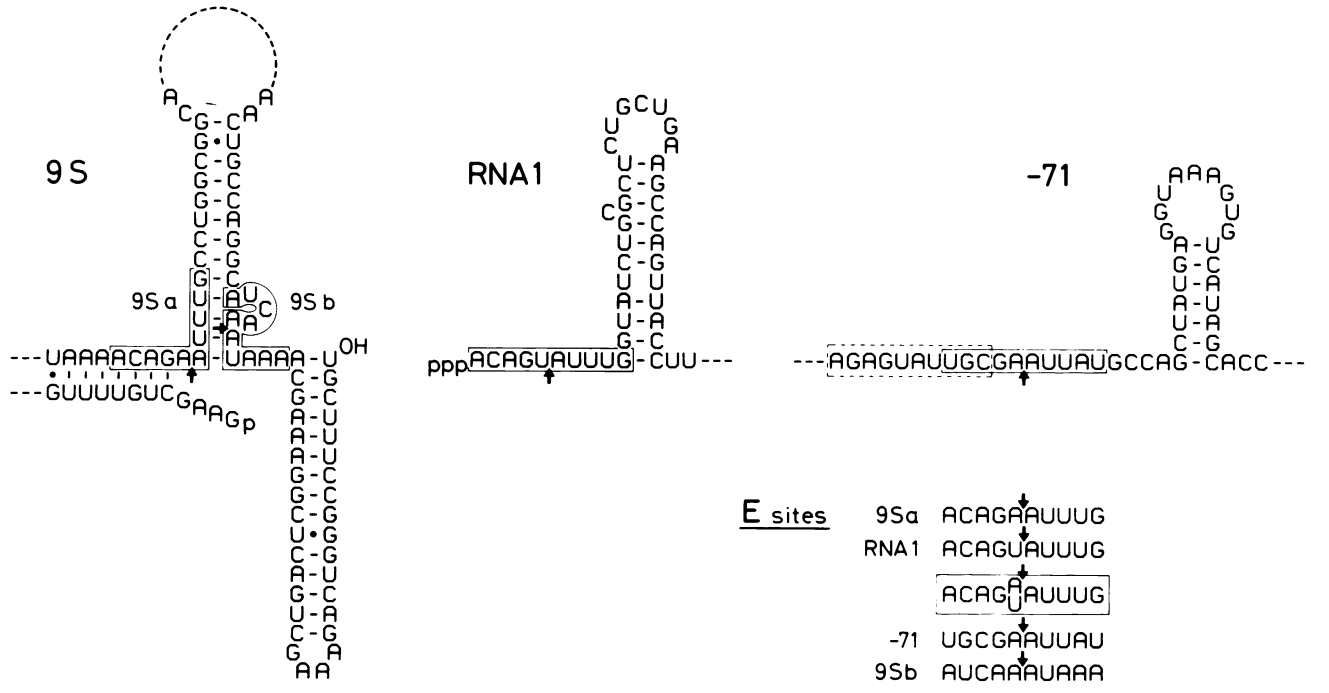

Fig. 6. A comparison of RNase E cleavage sites. Nucleotide sequences flanking the RNase E cleavage sites of the gene 32 −71 transcript, 9S RNA (Roy *et al.*, 1983) and RNA1 (Tomcsányi and Apirion, 1985), and the secondary structures close to the cleavage sites of these substrates, are shown. The structures of the 9S RNA and RNA1, of which only the double-stranded regions close to the RNase E cleavage sites are shown, are from Tomcsányi and Apirion (1985) and were originally determined by Roy *et al.* (1983) and Tamm and Polisky (1983) respectively. The structure of the gene 32 leader transcript is from Gorski *et al.*, 1985. There are two RNase E cleavage sites in the 9S RNA substrate which for this figure have been named the 9Sa and 9Sb sites. The 9Sa site is cut more efficiently than the 9Sb site (Roy *et al.*, 1983). The 5 nt on either side of the RNase E cleavage sites (arrowed) are boxed. A site upstream of the −71 processing site, which is similar to the E 'recognition sequence' (7/10), is indicated by a hatched box. It is conceivable that this upstream sequence is also recognized by RNase E but that processing at the downstream site prevented detection of its cleavage product. The nucleotide sequences flanking the E cleavage sites are aligned for comparison ('E sites'). A potential RNase E 'recognition sequence', based on the almost identical sequences at the 9Sa and RNA1 cleavage sites, is boxed (Tomcsányi and Apirion, 1985).

around the processing site. We have also included in Figure 6 the nucleotide sequences flanking the two RNase E cleavage sites in 9S RNA, '9Sa' and '9Sb'. The '9Sb' site, which is cut less efficiently than '9Sa' (Roy *et al.*, 1983), exhibits little similarity to the proposed RNase E 'recognition sequence' (4/10), but again the nucleotides immediately adjacent to the cleavage site (A/AU) are conserved. The potential secondary structures close to the RNase E cleavage sites are also shown in Figure 6. From these few examples, it seems possible that a combination of both nucleotide sequence immediately flanking the cleavage site and RNA conformation are required for RNase E recognition.

It has been suggested that *E.coli* processing ribonucleases can interact functionally with each other (Pragai and Apirion, 1982; Ray *et al.*, 1982; Gurewitz *et al.*, 1983) and there is evidence that they can co-sediment as a complex during ultracentrifugation (Jain *et al.*, 1982). In the case of the M1 RNA precursor for the RNA component of RNase P, this precursor accumulated in an RNase E-deficient strain but it was not cleaved by purified RNase E *in vitro* (Gurevitz *et al.*, 1983). One proposal to account for this finding was that RNase E is involved indirectly in the processing by affecting the activity of another enzyme. An indirect effect of RNase E processing on the production of mature T4 tRNAs has also been suggested (Pragai and Apirion, 1982). We have shown that neither RNase P nor III appear to have roles in the processing of the gene 32 mRNA. The simplest interpretation of our results is that RNase E itself processes the gene 32 mRNA, but *in vitro* studies are required to provide direct evidence for this.

Ribonuclease III processing of bacteriophage mRNA can affect its translation, as demonstrated in the case of the polycistronic message for T7 early genes (Dunn and Studier, 1975; Saito and Richardson, 1981) and the λp_L transcript (Schmeissner *et al.*, 1984). Some *E.coli* polycistronic mRNAs are also processed by RNase III (Barry *et al.*, 1980; Régnier and Portier, 1986) and RNase III processing of the messages for the polynucleotide phosphorylase gene has been shown to reduce the expression of this gene (Portier *et al.*, 1987). It will be interesting to find out whether gene 32 mRNA processing affects gene 32 expression. Previous experiments indicate that the processing may result in increased gene 32 expression. At late times after infection with a 55^- mutant phage, under conditions where transcription initiation had been inhibited early in infection (Krisch *et al.*, 1977), there was an increase in the synthesis of the gene 32 product (gp32). Since the processed species predominates under these conditions (Belin *et al.*, 1987), this suggests that the rate of gp32 synthesis from the processed transcript is greater than that from its precursors.

Processing of the gene 32 mRNA could potentially be regulated in concert with the infected cell's requirements for gp32. Such regulation of the processing could involve gp32, either directly or indirectly. It has been postulated that gp32 binds to its message in the A+T-rich region close to the ribosome-binding site and represses its translation (Krisch and Allet, 1982; von Hippel *et al.*, 1982). Interestingly, this A+T-rich sequence is adjacent to the stem−loop structure (Figure 2) which is near the RNase E processing site (Figure 6). It is possible that the binding of the gp32 to the gene

32 message could inhibit the processing reaction, for example by sterically hindering access to the E site. Preliminary results are compatible with regulation of processing by gp 32, since the level of processed message is much lower in phage infections where gene 32 translation is repressed.

The processing of the gene 32 mRNA results in the formation of a stable transcript, while the upstream sequences of the precursor messages decay (Belin *et al.*, 1987) in a manner analogous to that observed after processing at the intercistronic region of the polycistronic *papB–papA* message in the control of *E.coli* pilus gene expression (Båga *et al.*, 1988). The decay of the upstream sequences in the gene 32 mRNA could be initiated by the RNase E processing exposing 3′ ends which may then be targets for 3′ to 5′ exonuclease digestion. The stem–loop structure of the gene 32 terminator may have acted as a block to such decay prior to the processing event; it is known that stem–loop structures can stabilize RNAs in *E.coli* (see Brawerman, 1987). The stability of the gene 32 processed transcript could be due to the combination of stability determinants at its 5′ end (Gorski *et al.*, 1985) and the stem–loop structure at its 3′ end.

Gene 32 mRNA may not be the only mRNA to be processed by RNase E. Indeed, the pattern of protein synthesis in uninfected RNase E-deficient *E.coli* is different from that in the isogenic wild-type strain (Gitelman and Apirion, 1980; and this communication), suggesting that *E.coli* mRNAs could be affected by RNase E processing. A few examples of endonucleolytic cleavages affecting mRNA degradation have been found in *E.coli*, but the enzymes responsible have not yet been identified (Cannistraro *et al.*, 1986; Båga *et al.*, 1988; Melefors and von Gabain, 1988). It will therefore be interesting to examine other phage and bacterial transcripts for RNase E cleavages.

Materials and methods

Bacterial and phage strains

E.coli RNase E^+ and E^- isogenic strains N3433 (*rne*$^+$) and N3431 (*rne*$^-$3071 Ts) (Goldblum and Apirion, 1981), were obtained from B. Bachmann at the *E.coli* Genetic Stock Center (Yale University, USA). The RNase P-deficient (*rnp*A49) strain N2020 (Apirion, 1980) was from M. Belfort. U.Schmeissner provided the RNase III^+ and III^- isogenic pair AO159 *rnc*105 and AO160 *rnc*$^+$ (Schmeissner *et al.*, 1984); these had been constructed by A.Oppenheim from the *rnc*105 mutant of Studier (Studier, 1975) in the SA500 (Adhya *et al.*, 1974) background. The phenotypes of these RNase mutant strains were verified by analysing the stable RNAs labelled *in vivo* using [^{32}P]orthophosphate at the nonpermissive temperature (data not shown). The 30S (Nikolaev *et al.*, 1973) and 9S (Ghora and Apirion, 1979) rRNA precursors accumulated in the RNase III- and RNase E-deficient strains respectively, and the synthesis of mature tRNA was defective in the RNase P-deficient strain (Schedl and Primakoff, 1973). The *E.coli* strain MC1061 (Casadaban *et al.*, 1980) was also used as a T4 host. All of these *E.coli* strains were nonpermissive for the T4 amber mutant strains used.

Wild-type T4 (T4D), and the 55^- (55amBL292) and 32^-44^- (32amH18, 44amN82) mutant strains were from the Geneva collection.

The medium used for the growth of the *E.coli* strains N3431 and N3433 was M9S containing 0.2% Casamino acids (Champe and Benzer, 1962). Tris Minimal Medium (TMM) (Smith and Hedgpeth, 1975) supplemented with 20 μg/ml tryptophan was used for the growth of strains N2020, AO159 and AO160.

Bacterial growth and phage infection

Bacterial strains N3431 (*rne*$^-$) and N3433 (*rne*$^+$) were grown in M9S medium at 30°C under aeration to a cell density of $\sim 5 \times 10^7$/ml, centrifuged and resuspended in 1/8 volume of the same medium. These cultures, containing $\sim 4 \times 10^8$ cells/ml, were pre-incubated and aerated for 5 min before infection with a 1/10 volume of phage to give a final multiplicity of infection (m.o.i.) of 20. At 2 min after infection, the percentage of surviving bacteria was 10–20%.

The same method was used for strains N2020 (*rnp*$^-$), AO159 (*rnc*$^-$) and AO160 (*rnc*$^+$) except that they were grown in TMM medium to 2×10^8 cells/ml and resuspended at 4×10^8 cells/ml, then an m.o.i. of 10 phage was used. The percentage of surviving bacteria was <2% for the *rnp*$^-$ strain, ~20% for the *rnc*$^+$ strain and as high as 40% for the *rnc*$^-$ strain. Apirion and Watson (1975) had found that T4 had a reduced ability to infect an *rnc*$^-$ mutant strain of *E.coli*.

RNA isolation

Samples (2.5 ml) were removed at various times after infection and the RNA was extracted essentially as described by Hagen and Young (1978). RNase-free RQ1 DNase (Promega Biotech) was used to remove DNA.

5′-End mapping of transcripts by cRNA protection

The method and materials used have been previously described (Belin *et al.*, 1987). The plasmid pSP64TAK, containing the gene 32 leader region (from −343 to +3) cloned into the SP6 vector pSP64 (Melton *et al.*, 1984), was linearized with *Eco*RI (Boehringer) and used as a template in the *in vitro* transcription system as described. After hybridization of the synthesized ^{32}P-labelled cRNA to the total RNA, the samples were digested with 5 μg pancreatic RNase (Calbiochem)/μg total RNA. The protected hybrid RNAs were subsequently denatured and analysed by urea–PAGE. Gels were dried then exposed to X-ray film (Kodak X-OMAT 5) at −70°C with intensifying screens (Kodak).

5′-End mapping of transcripts by primer extension

The oligonucleotide primer 5′-TTAACAGTTGGTGCTATG was synthesized on an Applied Biosystems 381A instrument, deblocked, and used without further purification. This oligonucleotide is complementary to sequences from −28 to −45 relative to the gene 32 initiation codon (Figure 2).

RNA from phage-infected cells (1 μg) was hybridized to primer (10 ng) for 2 min at 70°C in 3 μl of 100 mM Tris–HCl pH 8.3, 125 mM NaCl, 12.5 mM $MgCl_2$ and 0.8 mM DTT. After 10 min at 25°C and 10 min at 0°C, the primer was extended for 10 min at 42°C in the presence of 8 U of AMV-reverse transcriptase (Promega), 8 U of placental RNase inhibitor (Promega), dCTP, dGTP, dTTP (50 μM each), 5 μCi of ^{32}P-labelled dATP (2.2 μM) and 4 mM DTT (final volume = 5 μl). After the addition of 5 μl of 80% formamide, 2 M urea, the samples were boiled and electrophoresed on a 10% polyacrylamide sequencing gel with the gene 32 sequencing ladder. For the dideoxy sequencing reactions, purified pRDB8 (Duvoisin *et al.*, 1986) plasmid DNA (1 μg), denatured for 5 min at 25°C in 0.4 M NaOH, was annealed to 70 ng of primer by co-precipitation in ethanol. Primer elongation was performed for 10 min at 42°C as described (Zagursky *et al.*, 1985).

Analysis of protein synthesis by pulse-labelling

Cells were grown and infected as described above. At time points equivalent to those used for the RNA isolation, 2.5-ml samples were removed and pulse-labelled with 2 μCi/ml ^{14}C-labelled amino acids (New England Nuclear NEC-445) for 3 min, followed by a 3-min chase with cold amino acids. The cell pellets were then lysed and their protein content analysed by SDS–PAGE (Laemmli, 1970). After staining of total proteins with Coomassie blue, the gels were autoradiographed at room temperature using Fuji RX X-ray film.

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